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Transmission of Hepatitis B to Chimpanzees by Hepatitis B Surface Antigen-Positive Saliva and Semen

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To assess the infectivity of hepatitis B surface antigen (HBsAg)-containing body fluids other than blood, chimpanzees were inoculated intravenously with saliva and semen obtained from HBsAg-positive individuals implicated in non-percutaneous transmission of hepatitis B. Saliva and semen samples were negative for occult blood. The titer of HBsAg in saliva was on the average only 1/3,000 that of the corresponding serum. One chimpanzee, inoculated sequentially with saliva from three individuals, developed HBsAg at 9 weeks and serum glutamic pyruvic transaminase elevation at 13 weeks after injection. HBsAg persisted for 15 weeks. This animal also developed e antigen, anti-core antibody, and anti-surface antibody. Liver biopsies showed acute hepatitis that subsequently resolved. A second chimpanzee, inoculated with HBsAg-positive semen, developed HBsAg and elevated serum glutamic pyruvic transaminase 4 weeks after inoculation and then died suddenly without explanation. HBsAg was positive in two consecutive samples and was confirmed by specific neutralization. Autopsy did not reveal evidence of hepatitis. This study demonstrates that HBsAg-positive saliva and, probably, semen contain infectious virus and suggests that saliva and/or semen may serve as important mechanisms in the transmission of type B hepatitis.

Hepatitis B surface antigen (HBsAg) has been detected in both saliva (4, 23) and semen (7) and, on epidemiological grounds, these secretions have been suggested as possible vehicles for hepatitis B transmission (17, 21). However, since only a minute portion of HBsAg-containing particles are infectious (6, 15), the presence of HBsAg does not establish the infectivity of a given material. In the absence of a tissue culture system, infectivity can only be directly assessed by the demonstration of hepatitis transmission to a susceptible animal. In this study, we have attempted to infect chimpanzees with HBsAg-positive saliva and semen derived from individuals clinically implicated in "non-percutaneous" transmission of type B hepatitis.

MATERIALS AND METHODS

Design of study. After base-line liver function studies and liver biopsy, HBsAg-positive saliva or semen was inoculated intravenously into a juvenile, seronegative chimpanzee. In the saliva transmission experiment, 1 ml of HBsAg-positive, occult blood-negative saliva was obtained from each of three patients (see case histories), and the individual samples of saliva were injected sequentially into separate veins of a single chimpanzee. An identical procedure was used in the semen experiment, except that 0.5 ml of semen from each of three individuals was utilized. Semen was obtained from two of the same individuals who provided saliva.

After inoculation, chimpanzees were sampled weekly for HBsAg, antibody to HBsAg (anti-HBs) and aminotransferase (serum glutamic pyruvic transaminase [SGPT] and serum glutamic oxalacetic transaminase) activity. If laboratory values indicated the onset of hepatitis and/or hepatitis B virus (HBV) infection, liver biopsies were obtained weekly and additional tests were performed, including e antigen and anti-core antibody (anti-HBc).

Chimpanzee 794 was born in captivity and was age 3 when utilized in the experiment. Chimp 23 (age 11) was wild-captured but had lived in captivity for 9 years at the time of experimentation.

Case histories. Details of the case histories are listed in Table 1. Patient 1 had acute lymphocytic leukemia and was a chronic carrier of HBsAg with intermittent and mild elevation of SGPT. One year after HBsAg was first detected, her mother developed acute HBsAg-positive hepatitis and her sister became anti-HBs positive. Epidemiological investigation (16) suggested that oral transmission of HBV was the most likely route of spread in this family.

TABLE 1. *Serological and histological data on patients providing saliva and semen for chimpanzee inoculations*

Source of inoculum (patient no.)	Age (yr)	Duration of HBsAg positivity (yr)	HBsAg subtype	Anti-HBs	Anti-HBc	e antigen	Liver biopsy		History of transmission to contact	Inoculum/chimp no.
							Histology	Fluorescence (HBsAg/HBcAg)		
1	11	2	ayw	−	−	+	CPH[a]	+/+	Yes	Saliva/794
2	25	1	ayw	−	−	+	CAH[b]	NT[c]	Yes	Saliva/794; semen/23
3	25	2	ayw	−	+	+	Mild portal inflammation	+/+	No	Saliva/794; semen/23
4	34	1	adw	−	+	+	CAH	+/+	Yes	Semen/23

[a] CPH, Chronic persistent hepatitis.
[b] CAH, Chronic active hepatitis.
[c] NT, Not tested.

Patient 2 was a dialysis technician when he developed HBsAg and then SGPT elevation (peak, 1,500 IU). He was anicteric and asymptomatic. A sexual partner of the patient developed icteric HBsAg-positive hepatitis, as did a technician accidentally inoculated with a needle contaminated with his blood. Saliva inoculated into chimpanzee 794 was obtained at the time of peak SGPT elevation. Semen was obtained 1 year after the onset of disease, when the SGPT was 116.

Patient 3 was a dialysis technician who developed anicteric HBsAg-positive hepatitis after an accidental needlestick; his infection progressed to a chronic HBsAg carrier state with persistent mild elevations of SGPT.

Patient 4 was a dental intern. When two of his patients developed HBsAg-positive hepatitis, he was tested and also found to be HBsAg positive. It could not be ascertained whether he had infected these patients or the converse. Two sexual partners of patient 4 subsequently developed acute, icteric, HBsAg-positive hepatitis. Serum SGPT was 1,000 when first tested and subsequently fluctuated in the 100 to 400 range. The semen sample inoculated into chimpanzee 23 was obtained 11 months after the onset of disease, at which time his SGPT was 231.

Test methods. HBsAg was tested by solid-phase radioimmunoassay (Ausria II, Abbott Laboratories) according to the manufacturer's directions. All positive results were confirmed by duplicate test, and specificity was established by neutralization with unlabeled anti-HBs. Results were expressed as the ratio of sample counts to the mean of negative control counts (*P/N* ratio), and a sample was considered positive when this ratio exceeded 2.1.

To determine the titer of HBsAg, serial twofold dilutions of neat serum or serum initially diluted 1:100 were tested by Ausria II. The end point of titration was the highest dilution that resulted in radioactive counts greater than 2.1 times the mean of negative control sera.

Anti-HBs was determined by both passive hemagglutination (22) of HBsAg-coated erythrocytes (Electronucleonics Inc.) and by solid-phase radioimmunoassay (AusAb, Abbott Laboratories). Anti-HBc was measured by an immune adherence test as described by Tsuda et al. (20).

Saliva samples (6 ml) to be tested for hepatitis B core antigen (HBcAg) and deoxyribonucleic acid (DNA) polymerase were layered onto 6 ml of 30% (wt/vol) sucrose in phosphate-buffered saline and centrifuged at 40,000 rpm for 5 h in an SW41 rotor. The pellet was suspended in 400 μl of TNB [0.01 M tris(hydroxymethyl)aminomethane, 0.1 M NaCl, 1 ng of bovine serum albumin per ml, pH 7.4] and sonically oscillated. A 10-μl volume of 10% Nonidet P-40 was added to 75 μl of the sonically treated pellet suspension, which was then tested for HBcAg by the method of Purcell et al. (16) and for DNA polymerase by the method of Kaplan et al. (9). The specificity of the polymerase reaction was determined by precipitation with anti-HBs as previously described (8). Salivas obtained from three HBsAg-negative individuals were pooled and used as the negative control. A positive control was prepared from HBsAg-positive serum having high hepatitis B-specific DNA polymerase activity. The serum was centrifuged at 20,000 rpm for 4 h, and the pellet was recentrifuged through 20% sucrose at 25,000 rpm for 5 h. A 100-μl portion of the final pellet suspension was added to 6 ml of HBsAg-negative saliva to serve as a positive control in assays for HBcAg and DNA polymerase.

e antigen and antibody were detected by a modified, double-immunodiffusion technique in agarose adapted from Williams and LeBouvier (24). The antibody employed in this test reacted with both the e_1 and e_2 components of e antigen, and the resulting precipitin lines were distinguished by their ability to form lines of identity with e_1 and e_2 reference antigens. Sections of liver tissue were snap-frozen in liquid nitrogen, sectioned on a cryostat, reacted with fluorescein-labeled guinea pig anti-HBs and human anti-HBc, and examined by fluorescence microscopy.

Occult blood was measured by the Hemoccult slide test (Smith Kline Diagnostics), which uses guaiac as the principle reagent, and by using N-Multistix (Ames Co.), which utilizes orthotolidine as the principle reagent. N-Multistix will detect

0.015 mg of hemoglobin per dl. If the initial test for occult blood was positive, the specificity of the reaction was established by the organic extraction of heme prior to testing for occult blood with a benzidine reagent. This extraction procedure avoids nonheme peroxidases, which can lead to false-positive results, and aqueous-based inhibitors such as ascorbic acid, which can lead to false-negative results. In the laboratory performing the test, the hemoglobin extraction procedure with benzidine was five times more sensitive than the N-Multistix method based on dilutions of a hemoglobin standard. (We are very grateful to Russell Jaffe for performing this test, which currently has a patent pending and which is in preparation for publication.)

Saliva collection. Unless otherwise noted, whole saliva, obtained without stimulation, was utilized in these experiments. In patients 1 and 2, saliva was also obtained directly from the secretion of the parotid gland. This was done by attaching a suction cup with integral cannula over the outlet of the parotid duct and collecting the secretion while the patient periodically touched lemon juice to the tongue. By using bilateral cannulae, approximately 1 ml was obtained per min. (We are indebted to Robert Wolf for performing this procedure.)

RESULTS

Characterization of inocula. Each of the saliva samples was negative for occult blood when tested by the Hemoccult (guaiac) method. However, two of the three samples of saliva were positive when tested by the N-Multistix (orthotolidine) method. When the orthotolidine-positive samples were subjected to a heme extraction procedure and the extract was then tested, the samples were negative for occult blood. Thus, the orthotolidine test yielded a false-positive reaction probably due to the presence of nonheme peroxidases in saliva.

The titer of HBsAg in saliva was determined by Ausria II and, in each individual, was compared with the HBsAg titer of serum obtained the same day. In patient 1, the comparative titers of saliva and serum were 1:16 and 1:12,800, respectively. Corresponding values for patient 2 were 1:2 and 1:12,800, respectively; those for patient 3 were 1:32 and 1:51,200, respectively.

DNA polymerase was measured in the saliva of patient 3 and in a pool of the three salivas utilized. High polymerase activity was detected but was not specifically precipitated by anti-HBs. In addition, a saliva pool obtained from three HBsAg-negative individuals demonstrated nonspecific elevation of DNA polymerase of similar magnitude. Although HBcAg was readily detected in the control saliva, to which HBcAg-positive material was added, it was not detected in either the saliva from patient 3 or in a pool consisting of saliva from patients 1, 2, and 3.

The saliva from patient 3 and the saliva pool were concentrated approximately 10-fold by pelleting in sucrose and were then examined by electron microscopy. The amount of background particulate matter was so great that it prohibited a valid examination. A small number of tubular forms was noted within the particulate debris; Dane particles were not observed.

Each of the salivas formed a precipitin line when reacted with anti-e antiserum. However, the line did not show a reaction of identity with the e antigen-positive control. In addition, similar reactions were noted in HBsAg-negative control salivas. The cause of these precipitin reactions has not been determined.

Parotid secretions were obtained from patients 1 and 2 and were HBsAg negative even though whole saliva obtained at the same time was HBsAg positive. Parotid saliva remained HBsAg negative after fourfold concentration.

All semen samples were negative for occult blood by the Hemoccult method. Only the semen from patient 2 was available in sufficient quantity to perform additional tests for occult blood; as with the saliva samples, it was positive by the orthotolidine method but negative by the specific extraction procedure.

There was an insufficient volume of semen to determine HBsAg titers or to perform tests for DNA polymerase or core antigen. As with saliva, contaminating particulate material in semen prevented accurate electron microscopy evaluation.

Saliva experiment. The response of chimpanzee 794 to intravenous injection of HBsAg-positive saliva is shown in Fig. 1. HBsAg first appeared 9 weeks after inoculation and then rose in concentration to a peak *P/N* of 44 in week 13. HBsAg subsequently declined until it was no longer detectable by week 25. HBsAg

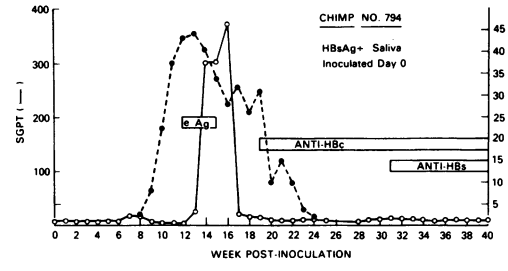

FIG. 1. *Clinical and serological response of chimp 794 to intravenous inoculation of HBsAg-positive saliva.*

was of subtype ayw, the same subtype as that of the three salivas inoculated. SGPT values were elevated from week 13 to week 17. The onset and peak of SGPT followed the corresponding appearance and peak of HBsAg by 4 weeks. The e_1 and e_2 components of e antigen became detectable in week 12, 4 weeks after the first appearance of HBsAg. The duration of detectable e antigen was 3 weeks compared with 15 weeks for HBsAg. Anti-HBc first appeared in week 19, when HBsAg was rapidly declining, and anti-HBs first became detectable in week 31, 7 weeks after the disappearance of HBsAg.

Chimpanzee 794 did not become clinically ill. Liver biopsies obtained weekly beginning in week 10 were typical of acute viral hepatitis; subsequently, biopsies revealed complete resolution of hepatic abnormalities. Immunofluorescence studies performed on the liver biopsy obtained at week 11 were negative for HBsAg and HBcAg.

Semen experiments. Four weeks after intravenous inoculation of HBsAg-positive semen, chimpanzee 23 had a rise in SGPT value to 75 from a base-line value of 11 and simultaneously had the first appearance of HBsAg (P/N = 2.4). Neutralization tests demonstrated HBsAg specificity. Two days later, the chimpanzee suddenly died, manifesting signs of shock but no symptoms suggesting fulminant hepatic failure. Necropsy did not reveal the cause of death, and blood cultures were negative. Histological examination of liver biopsy obtained at necropsy revealed marked hepatic congestion but no evidence of hepatitis. Immunofluorescence studies performed on a liver biopsy obtained 2 days prior to death were negative for HBsAg and HBcAg.

A blood sample drawn at necropsy was also positive for HBsAg (P/N = 4.3), and specific neutralization was again demonstrated. HBsAg could not be subtyped because of the weak reaction. The two HBsAg-positive samples were negative for e antigen and negative for anti-HBc and anti-HBs.

DISCUSSION

There is considerable inferential evidence suggesting that HBV may be transmitted by routes other than percutaneous inoculation. This evidence includes: (i) the failure to elicit a history of blood transfusion or other percutaneous inoculation in a large proportion of patients hospitalized with type B hepatitis (14); (ii) the intrafamilial spread of HBV infection (19); (iii) the transmission of HBV among unrelated sexual partners (25); (iv) the prevalence of anti-HBs among volunteer blood donors, which suggests a minimal hepatitis B infection rate in the United States of 7 to 14% (5, 11); and (v) the very high prevalence of HBsAg and anti-HBs among institutionalized patients (18) and among populations of low socioeconomic status (3). These observations are further supported by the finding of HBsAg in body fluids other than blood (7, 13, 23) and by the association of HBsAg-positive saliva and/or semen with transmission of HBV in several epidemiological investigations (17, 21, 25).

The presence of HBsAg does not, however, establish the infectivity of a given substance, since most of this antigen is associated with subviral particles or defective viral particles that appear to be noninfectious (6, 15). Infectivity should correlate directly with the number of complete HBV particles (complete Dane particles) and only indirectly with the presence of HBsAg. The final link, then, would be to demonstrate complete Dane particles in these secretions or, more conclusively, to document their infectivity in a susceptible animal. This study, in fact, demonstrated that saliva and, probably, semen samples were infectious for chimpanzees and, by inference, supports the theory that these secretions may be important vehicles for hepatitis B transmission. Chimpanzee 794 developed classical type B hepatitis. The development of type B hepatitis in chimpanzee 23 was less well established because the animal died unexpectedly 4 weeks after inoculation. The cause of death remains unknown, but it did not appear related to hepatitis. The development of HBV infection is based solely on the presence of two consecutive serum samples demonstrating low-level, but specific, HBsAg and a coincident rise in the SGPT just before death.

We did not demonstrate in this study that saliva and semen are infectious by their natural routes, namely, by oral and/or venereal contact. We considered this secondary to the direct demonstration of the infectivity of HBsAg-positive saliva and semen. Recently, Bancroft et al. (1) infected two gibbons by the subcutaneous administration of HBsAg-positive saliva but were unable to transmit hepatitis when the same inoculum was given by intranasal or intraoral routes. The failure of saliva to infect by these routes may be a function of the amount of virus given. We have shown in this study that the titer of HBsAg is markedly lower in saliva than in the corresponding serum. The number of infectious particles may be correspondingly low. It would be of interest to determine, in additional animals, whether

saliva and semen with a high titer of HBsAg, a large number of Dane particles, and/or a large amount of hepatitis B-specific DNA polymerase can transmit hepatitis B infection by intraoral, intravaginal, or intrarectal inoculation. The aforementioned epidemiological studies (19, 21, 25) suggest that such transmission is probable, as does an earlier study which demonstrated that HBsAg-positive serum was infectious when given orally (8). In addition, the development of hepatitis B after a human bite has been reported (12).

Although we were unable to demonstrate the presence of Dane particles, hepatitis B-specific DNA polymerase, or core antigen in the saliva by in vitro assays, this material was infectious when biologically tested. This was not unexpected since a biological assay of HBV has been shown to be extremely sensitive (2). However, there were also technical problems that interfered with a detailed characterization of the salivary inoculum. The large amount of nonviral particulate matter in saliva hampered electron microscopy observations, and the presence of other polymerases, probably bacterial in origin, interfered with interpretation of the assay for DNA polymerase.

There was a marked difference in the titer of HBsAg in saliva as compared with the corresponding titer in serum; serum titers were, on the average, 3,000 times that of saliva. In comparsion, the *P/N* ratio of undiluted serum was, at most, eight times that of the corresonding saliva; hence, the *P/N* ratio in the solid-phase radioimmunoassay can be used only as a crude quantitative estimate of HBsAg.

In this experiment, we specifically tested parotid secretions as compared with whole saliva and consistently found the former to be HBsAg negative and the latter to be HBsAg positive. This indicates that HBsAg-containing particles are not actively secreted by the parotid gland but, rather, that they gain entrance into the saliva either throught the sublingual or submaxillary salivary glands or, more likely, through the buccal mucosa. These experiments do not elucidate the mechanism by which the latter might occur.

For the present, one can assume that, if a patient's serum contains infectious particles, then his other body fluids, particularly saliva and semen, may also contain infectious particles. These secretions may serve as important vehicles in hepatitis B transmission.

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